ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1985, p. 695–700
0066-4804/85/050695-06$02.00/0

Vol. 27, No. 5

Response of *Legionella pneumophila* to β-Lactam Antibiotics

STEVEN WEISHOLTZ AND ALEXANDER TOMASZ*
The Rockefeller University, New York, New York 10021

Received 18 October 1984/Accepted 5 February 1985

Legionella pneumophila **Philadelphia strain 1 grown in vitro contained five penicillin-binding proteins that were accessible to the antibiotic in membrane preparations and in live cells as well. The bacterium had reasonably low MICs of several β-lactam antibiotics and was susceptible to both the bactericidal and the lytic activity of these drugs. An unusual feature of the response of this bacterium to penicillin treatment was that cell lysis as determined by decrease in culture turbidity and release of intracellular macromolecules was not accompanied by degradation of the peptidoglycan.**

Legionella pneumophila, although similar to other gram-negative bacteria in terms of its basic cell envelope structures and composition (1, 8, 13), has several unusual characteristics. It was reported that cell wall preparations from *L. pneumophila* Philadelphia strain 2 contain strongly bound protein material that is not removed by trypsin but only by alkali treatment, and that cleavage of the glycan chains in the *L. pneumophila* wall by egg white lysozyme does not cause a decrease in the turbidity of cell wall suspensions (1). Because an unusual cell wall structure may contribute to the relative inefficacy of β-lactam antibiotics against these pathogens (2, 6, 9) and because the physiology of interactions of *L. pneumophila* with these antibiotics has not been well documented, we undertook a morphological and physiological study of the response of this bacterium to some β-lactam antibiotics. We showed that *L. pneumophila* has penicillin-binding proteins (PBPs) similar to those found in other gram-negative bacilli. The bacteria had prompt bactericidal and lytic responses to various β-lactam antibiotics. However, these responses were unique in that penicillin-induced cell lysis was not accompanied by extensive cell wall degradation.

MATERIALS AND METHODS

Strains and media. *L. pneumophila* Philadelphia strain 1, obtained from Marcus Horwitz, the Rockefeller University, New York, N.Y., was used in all experiments. The organism was maintained by subculturing onto charcoal-yeast extract agar (7) every 72 h after growth at 37°C with 5% CO_2. For growth experiments, several colonies were selected from the agar plate and inoculated into a synthetic medium (15) that was supplemented with yeast extract (0.1%; Difco Laboratories, Detroit, Mich.), bovine serum albumin (0.4%), L-cysteine (0.4 mg/ml), and ferric pyrophosphate (0.25 mg/ml). This medium, to be referred to as growth medium in this paper, was easy to prepare and supported luxuriant growth of *L. pneumophila* in preliminary experiments. In labeling experiments with glucosamine, the basic medium was used except that all glucose was omitted. The *Escherichia coli* strain used was W7 (*lys dap*).

Antibiotics and reagents. Benzylpenicillin G (Bristol Laboratories, Syracuse, N.Y.), aztreonam (E. R. Squibb & Sons, Princeton, N.J.), thienamycin, mecillinam, cefotaxime, and cefoxitin (Merck & Co., Inc., Rahway, N.J.) were stored as powders and then dissolved in water at the time of an experiment. Other chemicals, reagents, and medium components were reagent-grade, commercial products.

MIC assays. All MIC assays were done in growth medium with a broth macrodilution method. Twofold serial dilutions of the antibiotics were made in broth on the day of the experiment. The organism had been subcultured from a charcoal-yeast agar plate into 10 ml of growth medium and grown overnight at 35°C with aeration. A 1:10 dilution of this culture was made into fresh, prewarmed medium and then grown back over a period of 4 to 5 h to log phase. Another 1:10 dilution was then made, and 0.1-ml portions of this diluted culture were inoculated into the antibiotic tubes containing a final volume of 1 ml. The final inoculum was $\sim 10^5$ CFU per MIC tube. Purity of the cultures was ascertained by microscopic and colonial appearance and failure to grow on tryptic soy agar and blood agar plates. The MIC tubes were incubated at 37°C with 5% CO_2, and growth was assessed at 48, 72, and 96 h. Samples (100 μl) were removed, serially diluted, and plated for the determination of MBCs, defined as the lowest antibiotic concentration that gave a 99.9% kill after 48 h growth. Colony counts were done after plating onto charcoal-yeast agar and incubating at 37°C with 5% CO_2 for 72 h.

Growth and lysis experiments. The organism was subcultured from an agar plate into 10 ml of growth medium and grown overnight at 37°C with aeration. The culture was then backdiluted into fresh medium and regrown to log phase. Growth and lysis after addition of antibiotic was monitored by using a Coleman nephelometer with a 620-nm filter. One hundred nephelometric turbidity units corresponded to $\sim 10^7$ CFU/ml. Cell viability was determined by making dilutions in growth medium and then plating onto charcoal-yeast agar plates.

Morphology. To assess the morphological response to antibiotics, 1 ml of a log-phase culture containing $\sim 10^7$ CFU/ml was dispensed into sterile tubes, and antibiotics were added in concentrations of 0.25, 0.50, 1, and 10× MICs. The cultures were incubated at 37°C with 5% CO_2 for 24 h, and then phase microscopy photographs were taken with a Nikon camera and Carl Zeiss microscope at ×1,250 magnification. Cultures were prepared for electron microscopy by adding glutaraldehyde (2%) and by chilling on ice for 10 min. The cultures were then filtered over a 45-μm Millipore membrane filter (Millipore Corp., Bedford, Mass.). The filter was placed in 2% glutaraldehyde with 0.1 M KPO_4 buffer (pH 7.0) to fix the cells. Dehydration, embedding, and thin sectioning were done as described before (17).

Peptidoglycan degradation and cell lysis. Log-phase cul-

* Corresponding author.

tures of *L. pneumophila* were labeled by growth for 18 to 20 h in 10-ml batches of [^{3}H]glucosamine (19.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) at 2 μCi and 2 μg of medium per ml and no glucose. The cells were then centrifuged at 7,500 rpm at 4°C and suspended in 50 ml of prewarmed medium to which 50 μg of nonradioactive glucosamine per ml had been added. The culture was then incubated for 4 to 5 h to ensure utilization of intracellular label. Such prelabeled, exponentially growing cultures received the antibiotics at various concentrations, and incubation continued. At intervals, 1.0-ml samples were removed and centrifuged in an Eppendorf microfuge at 4°C for 10 min. The supernatant was discarded, and the pellet was suspended in cold H_2O, transferred to borosilicate tubes, and frozen. After thawing, the samples were boiled in 4% sodium dodecyl sulfate (SDS) for 30 min. The SDS-insoluble material was washed three times with H_2O over a Millipore filter (0.45-μm pores). The filters were then placed in vials with 1 ml of potassium phosphate buffer (50 mM, pH 7.0) containing 200 μg of M1 muramidase and were gently agitated at room temperature overnight. This procedure resulted in the complete solubilization of radioactively labeled cell walls. Portions of the solutions were assayed for radioactivity in 15 ml of liquid scintillation fluid (Ultrafluor; National Diagnostics, Somerville, N.J.) in a Nuclear-Chicago Mark II scintillation counter.

For RNA labeling, log-phase cultures were labeled during overnight growth with a combination of [^{3}H]uracil (230 Ci/mmol; New England Nuclear) at 5 μg and 2.5 μCi of medium per ml. The cells were centrifuged, suspended in fresh medium, and grown for 4 h to allow utilization of intracellular label. After the addition of antibiotic, 500-μl portions were removed and centrifuged in an Eppendorf microfuge for 10 min at 4°C. Radioactivity released from the cells during antibiotic treatment was determined. A 250-μl portion of the supernatant was added to an equal volume of ice-cold 10% trichloroacetic acid and kept at 4°C overnight. The trichloroacetic acid-precipitable material was collected on Millipore filters (0.45-μm pores), washed three times with H_2O, and dried at 100°C for 1 h. A toluene-based fluor was added to the filters, and radioactivity was determined as above. Total radioactivity was determined by trichloroacetic acid treatment of a 500-μl sample of cells before antibiotic exposure.

Preparation of murein labeled with radioactive glucosamine. Cultures were prelabeled with [^{3}H]glucosamine as above. After harvesting by centrifugation, the cells were suspended in H_2O and frozen. After thawing, the cells were boiled in 4% SDS for 30 min with the addition of 50 mM sodium acetate (pH 5.0) to minimize loss of *O*-acetyl groups (3). After cooling, the SDS-insoluble material was collected by ultracentrifugation at 100,000 × *g* for 30 min. The pellet was washed with H_2O three times by ultracentrifugation. For muramidase digestion assays, 100-μl samples of purified peptidoglycan containing ~10,000 cpm were added to 1 ml of buffer and various enzymes and incubated overnight. The following enzymes were used: trypsin (in 100 mM Tris, pH 8), crude M1 muramidase (in 100 mM potassium phosphate, pH 7), purified M1 muramidase (in 100 mM potassium phosphate, pH 7), or egg white lysozyme (in 50 mM ammonium acetate, pH 6.5) at 100 μg per ml or Chalaropsis muramidase (20 μg/ml). The buffers used were Tris, KPO_4, and sodium acetate (50 mM, pH 5.0) for Chalaropsis muramidase. The digested samples were boiled in 4% SDS for 30 min. The SDS-insoluble portion was then removed by Millipore filtration (0.45-μm pores). The solubilized material was analyzed by thin-layer chromatography (TLC). Undigested murein was quantitated by determining the radioactivity on the filters.

TABLE 1. MICs and MBCs of several β-lactam antibiotics for *L. pneumophila* Philadelphia strain 1

Antibiotic	MIC_{48}[a]	MIC_{72}	MBC_{48}
Benzylpenicillin	1.25	1.25	1.25
Aztreonam	5.0	5.0	10.0
Thienamycin	≤0.02	0.02	≤0.02
Mecillinam	1.25	10	>10
Cefotaxime	0.15	0.15	0.15
Cefoxitin	≤0.02	0.15	0.3

[a] MIC_{48}, MIC after 48 h of exposure to antibiotic as described in the text.

TLC. The procedure of Gmeiner and Kroll was used (10). Solubilized peptidoglycan (8,000 to 20,000 cpm) was applied to a 0.25-mm-thick silica gel-coated plastic sheet (20 × 20 cm; Eastern Organic Chemicals, Rochester, N.Y.). The TLC plate was activated before sample application at 100°C for 30 min. The chromatogram was developed twice in isobutyric acid–1 M ammonia (5:3, vol/vol), dried, and sprayed with En^{3}Hance (New England Nuclear), and the fluorographs were exposed for 6 days. Radioactive spots were cut out, placed in scintillation vials, and counted in a toluene-based fluor.

PBPs. After overnight growth, the culture was backdiluted 1:10 into fresh broth and grown to log phase. This culture was concentrated 10-fold, and 100-μl volumes were exposed to various concentrations of [^{3}H]benzylpenicillin (25 Ci/mmol; Merck & Co.) and incubated for 30 min at 37°C. The cultures were then chilled, centrifuged at 4°C for 10 min in an Eppendorf microfuge, and washed twice with 1 ml of cold potassium phosphate buffer (50 mM, pH 7.0) to remove the albumin containing medium. The sediment was suspended in 50 μl of potassium phosphate buffer; 35 μl of sample buffer was added, and the samples were boiled for 5 min (4). They were subjected to polyacrylamide gel electrophoresis (PAGE) for the detection of PBPs as previously described (16). Fluorograms were exposed for 12 days.

L. pneumophila membranes were prepared from 50-ml cultures grown overnight. After sedimentation at 4°C, the cells were washed three times in 20 ml of potassium phosphate buffer (50 mM, pH 7.0), suspended in 5 ml of H_2O containing 4 mM $MgCl_2$, and frozen at −20°C. After thawing, an equal volume of glass beads (100-μm diameter) was added, and the mixture was shaken on a Mickle disintegrator for 60 min at 4°C. DNase and RNase (both 50 μl at 2 mg/ml) were added, and the mixture was incubated for 10 min at 37°C. The suspension was then centrifuged at 5,000 × *g* for 10 min, and the supernatant was collected and ultracentrifuged at 40,000 rpm for 40 min at 4°C. The sediment was washed three times in H_2O, suspended in 1.2 ml of H_2O, and frozen (1.3 mg of protein per ml).

For the PBP assay, 260 μg of membrane was incubated with various concentrations of [^{3}H]benzylpenicillin for 20 min at 37°C. The reaction was stopped by adding 10 μl of Sarkosyl and 5 μl of nonradioactive benzylpenicillin (12.5 μg/μl). Next, 40 μl of sample buffer was added, and the mixture was boiled for 5 min. The sample was then subjected to PAGE, and fluorograms were prepared as above.

For the determination of the rates of deacylation of penicilloyl-PBPs, the membrane preparation was incubated for 20 min with a saturating concentration of [^{3}H]benzylpenicillin (20 μg/ml) followed by the addition of nonradioactive

TABLE 2. Ability of β-lactam antibiotics to cause lysis of *L. pneumophila*[a]

Time[b] (h)	% of initial turbidity				
	Penicillin G	Thienamycin	Mecillinam	Aztreonam	Control
0	100	100	100	100	100
0.5	100	100	100	100	110
1	70	90	70	95	130
1.5	55	60	55	80	165
2	50	50	50	60	180
4	45	45	50	55	210

[a] When exponentially growing cultures, with shaking, reached a cell concentration of ~5 × 10^7 CFU/ml, antibiotics were added, each at a concentration of 10× MIC, and the turbidity was monitored with a Coleman nephelometer.

[b] Time after addition of antibiotic.

benzylpenicillin (2.5 mg/ml), and incubation at 37°C continued. Portions (70-μl) were removed after various intervals and the reaction was stopped with Sarkosyl. Samples were then subjected to PAGE as described above.

RESULTS

The MICs and MBCs of several selected β-lactam antibiotics for *L. pneumophila* Philadelphia strain 1 are shown in Table 1. Under the conditions of the assay, the organisms grew very slowly in both antibiotic and control tubes. Therefore, the MIC tubes were read after 48 h and again after 72 h of growth. For all antibiotics tested the MBCs differed from the 72-h MICs by at most one dilution tube. At antibiotic concentrations of 10× MIC, there was prompt lysis of *L. pneumophila* cells as indicated by the rapid loss of culture turbidity (Table 2). In general, benzylpenicillin and mecillinam caused the most rapid loss of turbidity, followed by thienamycin and then aztreonam. A decrease of approximately 50% in initial turbidity occurred after 2 h of exposure to each antibiotic except aztreonam. The latter did not achieve the same degree of lysis until after 4 h of exposure. On the same time scale the lytic response of *L. pneumophila* after exposure to penicillin was delayed in onset when compared with that of *E. coli* cultures (14).

Alteration of pH of the medium from 6.4 to 7.4 did not affect the ability of penicillin G to promote cell lysis. Below pH 6.4 growth of the organism was inadequate to allow experimentation.

Loss of viability of *L. pneumophila* after exposure to β-lactam antibiotics paralleled the decline in turbidity (Fig. 1). All antibiotics investigated were bactericidal. Aztreonam caused a slower decline in CFUs than the other β-lactams tested, and a 99.9% loss of viability was achieved at 24 h with an antibiotic concentration of 8× MIC. In contrast, penicillin G at 1× MIC achieved a comparable decrease in viability at 24 h. At sub-MICs (e.g., 0.5× MIC) we often observed an initial decline in viability at 24 h, with regrowth of the culture occurring upon longer incubation. When tested, these organisms had the same MIC as the original inoculum, an indication that resistance did not develop after penicillin exposure at sub-MICs.

To further investigate the physiology of the lytic response of *L. pneumophila* to β-lactam antibiotics, a series of labeling experiments was done. Cells were labeled with [^{3}H]uracil and then exposed to lytic doses of benzylpenicillin (25 μg/ml) (Fig. 2). Release of radioactively labeled nucleic acids into the medium was assayed by determining trichloroacetic acid-precipitable counts in the supernatant after centrifugation. As can be seen, significant amounts of nucleic acids were liberated into the surrounding medium parallel with the decline in culture turbidity. Over 50% of the macromolecular uracil label was released from the penicillin-treated cells in 3 h.

Despite the significant loss of turbidity and viability and release of intracellular macromolecules during penicillin-induced cell lysis, there seemed to be very little accompanying cell wall degradation. Figure 2 shows the amount of [^{3}H]glucosamine remaining in intact cell wall polymers (SDS-insoluble material) during a 4-h exposure to benzylpenicillin. There was little, if any, decline in the SDS-insoluble fraction of radioactivity after penicillin-induced lysis of *L. pneumophila*. Subsequent exposure of the penicillin-treated cells to M1 muramidase for 4 h caused an 80% loss of SDS-insoluble counts, further supporting the lack of cell wall degradation by penicillin alone (data not shown).

The lack of peptidoglycan degradation during penicillin-induced lysis was also consistent with the retention of

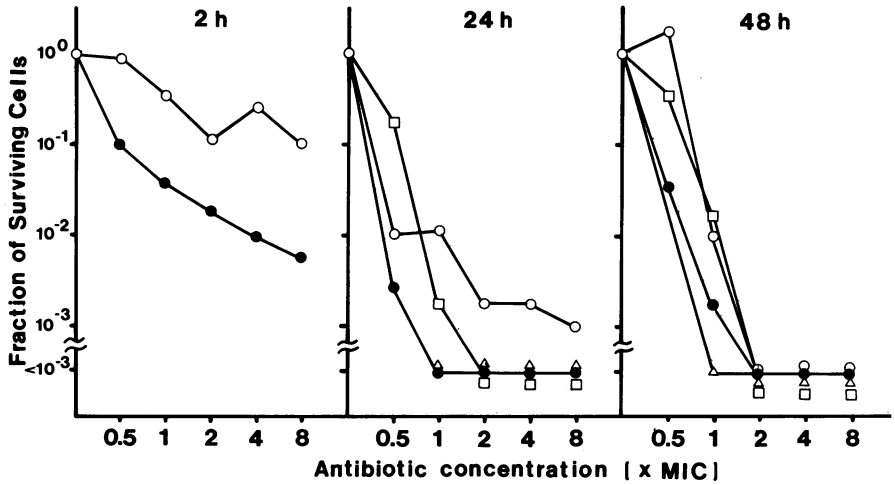

FIG. 1. Bactericidal activity of various β-lactam antibiotics. Cultures were grown at 37°C with 5% CO_2 without shaking. In the exponential growth phase, cultures were diluted to an inoculum concentration of 3 × 10^5 CFU/ml, and antibiotics were added at the concentrations (multiples of the MIC) indicated. Viability was determined as described in the text. Symbols: ●, benzylpenicillin; ○, aztreonam; □, cefoxitin; △, thienamycin.

bacillary morphology after antibiotic exposure. Electron micrographs (not shown) of *L. pneumophila* after penicillin-induced lysis demonstrated only a general decrease in cell contents (decrease in electron-dense material) that corresponded to the decrease in turbidity and release of RNA and cell contents into the medium. The lack of correlation between cell turbidity and intactness of peptidoglycan was also demonstrated in experiments in which [^{3}H]glucosamine-labeled *L. pneumophila* was treated with 1% SDS or with M1 muramidase. In the former case, 80% of turbidity was lost without any solubilization of peptidoglycan label. In contrast, a virtually complete degradation of peptidoglycan by M1 muramidase was accompanied by only about a 20% drop in turbidity.

Isolated murein sacculi labeled with [^{3}H]glucosamine from *L. pneumophila* appeared to be susceptible to degradation by exogenous muramidases despite the inability of benzylpenicillin to induce cell wall degradation in vivo. Table 3 presents the results of overnight peptidoglycan digestion by various murein hydrolases. As can be seen, the *N,O*-diacetyl muramidases, such as the Chalaropsis muramidase and purified M1 muramidase, caused a 95 to 98% loss of SDS-insoluble counts, indicating near total degradation of the murein sacculi. Trypsin treatment did not release any radioactive label from the SDS-insoluble murein, and egg white lysozyme produced only a 64% degradation.

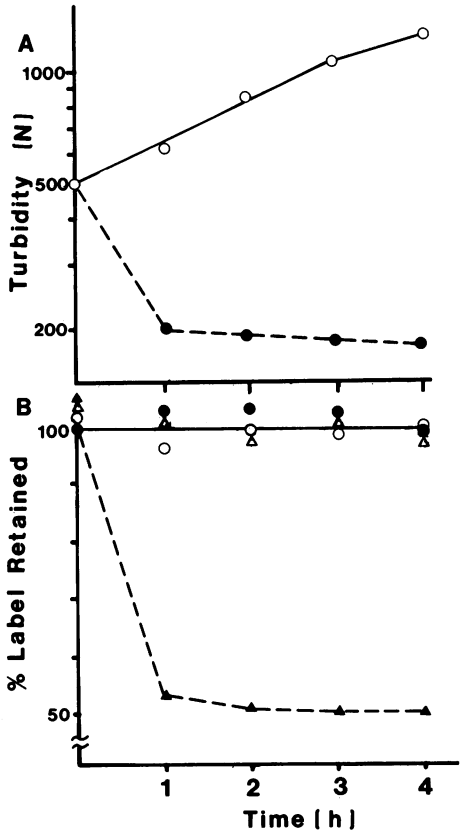

FIG. 2. Penicillin-induced lysis of *L. pneumophila* and its effect on release of cell wall and intracellular material. (A) Loss of turbidity after exposure to lytic doses of benzylpenicillin (25 μg/ml) was determined by nephelometry. Symbols: ●, benzylpenicillin; ○, control. (B) Release of [^{3}H]uracil-labeled nucleic acids from the intracellular pool and [^{3}H]glucosamine label from the SDS-insoluble cell wall fraction after exposure to lytic doses of benzylpenicillin (25 μg/ml) was determined as indicated in the text. Data are expressed as percentages of radioactive label remaining in the intracellular pool or SDS-insoluble cell wall fraction, respectively. Each point represents a mean of two experiments. Symbols: ▲, [^{3}H]uracil, penicillin exposure; △, [^{3}H]uracil, control; ●, [^{3}H]glucosamine, penicillin exposure; ○, [^{3}H]glucosamine, control.

TABLE 3. Hydrolysis of murein sacculi by various exogenous muramidases[a]

Muramidase (μg/ml)	Murein hydrolysis (%)
Pure M1 muramidase (10)	95
Chalaropsis enzyme (10)	98
N-Acetyl muramidase (100)	96
Lysozyme (100)	64
Trypsin (100)	0

[a] [^{3}H]glucosamine-labeled cell walls (5×10^3 cpm and 50 μg/100 μl) were incubated with a variety of enzymes in reaction mixtures of 1-ml volumes made up of 100 μl of cell wall substrate and 900 μl of the following buffers: 100 mM potassium phosphate at pH 7.0 in the cases of M1 and *N*-acetyl muramidase, at pH 5.0 for the Chalaropsis enzyme, and at pH 6.5 for egg white lysozyme. Tris (pH 8.0; 100 mM) was used with trypsin. The mixtures were incubated for 12 h at room temperature, and the fraction of SDS-insoluble counts was determined as described in the text. The degree of murein hydrolysis was expressed in relation to an undigested control.

TLC of Chalaropsis-treated murein sacculi confirmed the virtually complete degradation of the *L. pneumophila* cell wall by exogenous muramidase (Fig. 3). A similarly digested peptidoglycan of *Neisseria gonorrhoeae* was also chromatographed for comparison (courtesy of T. Dougherty, Rockefeller University). More than 96% of the original radioactive glucosamine label migrated either as the disaccharide peptide monomer or as the bis-disaccharide peptide, and no *O*-acetylated components were detectable by this method.

To further elucidate the interaction of β-lactam antibiotics with *L. pneumophila*, we investigated the PBPs on the bacterial inner membranes. After in vivo labeling of log-phase cultures with tritiated penicillin, we assayed PBPs in an SDS-PAGE system. Five binding proteins were consistently detected (Fig. 4). Their respective molecular weights

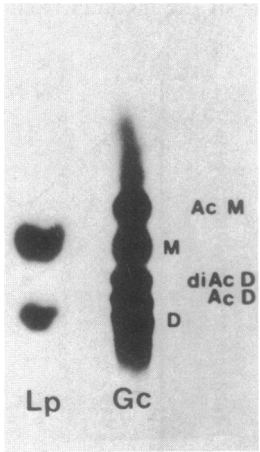

FIG. 3. TLC of the [^{3}H]glucosamine-labeled murein fragments generated by digestion of *L. pneumophila* (Lp) cell walls with the Chalaropsis muramidase. A total of 8,000 cpm of enzyme hydrolysate was spotted on the chromatogram. Also shown are the muramidase degradation products of gonococcal peptidoglycan (Gc) (5). The digested fragments include mono-*O*-acetylated monomers (AcM), monomers (M), di-*O*-acetylated dimers (diAcD), mono-*O*-acetylated dimers (AcD), and dimers (D).

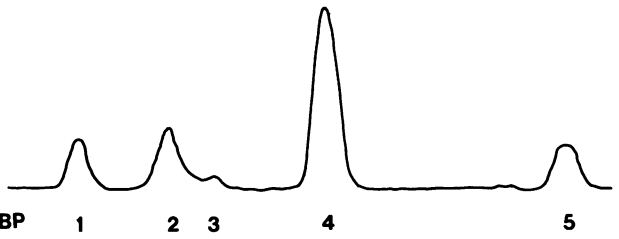

FIG. 4. PBPs of *L. pneumophila*. *L. pneumophila* cells were treated with 6.25 μg of [^{3}H]penicillin per ml, and the PBPs were separated by SDS-PAGE followed by fluorography, as described in the text. The fluorograms were scanned with a Quick Scan Junior densitometer (Helena Laboratories, Beaumont, Tex.).

were 95,000, 71,000, 61,000, 40,000, and 19,000. PBP 2 was occasionally resolved into two bands migrating at molecular weights of ~70,000 and ~64,000. Pretreatment of the cells with nonradioactive penicillin, Sarkosyl, or boiling prevented binding of [^{3}H]penicillin to these binding proteins. Incubation of prelabeled membrane preparations with 0.2 M neutral hydroxyamine for 20 min caused the loss of most of the bound penicillin from the PBPs. PBP 4 bound the greatest amount of label (57%), followed by PBPs 1 and 2 (15 to 18%). However, PBPs 1 and 2 appeared to have the highest affinity for [^{3}H]penicillin, with labeling at a concentration of 0.1 to 0.5× MIC for the organism. PBP 3 appeared with 1 to 2× MIC, and PBPs 4 and 5 did not appear until a concentration of 5 to 10× MIC was used.

In vitro labeling with [^{3}H]penicillin of membrane preparations from *L. pneumophila* demonstrated a similar PBP pattern. No loss of label from any of the penicilloyl-PBPs could be demonstrated during 60 min after postincubation with nonradioactive penicillin.

DISCUSSION

The *L. pneumophila* strain examined exhibited several of the typical responses shown by gram-negative bacilli treated with β-lactam antibiotics. The organism contained five PBPs accessible to penicillin both in membranes and in the growing cell. Treatment with aztreonam or mecillinam resulted in a tendency to form elongated or spheroid cells, respectively (undocumented finding). This is reminiscent of the behavior of *E. coli* and other gram-negative bacilli and suggests the presence in *L. pneumophila* of PBPs with selective affinities for certain β-lactam antibiotics (16). Both morphological changes required prolonged incubations with the drugs, presumably because of the long doubling time (3 to 4 h) of these bacteria (7, 19).

Cell walls (sacculi insoluble in hot SDS) of this *L. pneumophila* strain labeled with radioactive glucosamine could be fully degraded by treatment with either the *Streptomyces globus* M1 muramidase or the Chalaropsis muramidase (5). In analysis by TLC (10), over 96% of the degraded material was found to have migrated as a mixture of the disaccharide peptide monomer (about 60%) and the bis-disaccharide peptide dimer (30%). A small percentage of the radioactivity migrated in the region of higher oligomers. There was no evidence for *O*-acetylated muramyl derivatives. With the data from two representative experiments, the percentage of cross-linking between stem-peptides of the peptidoglycan of this *L. pneumophila* strain may be calculated as 19 to 25% (i.e., [(0.5 × the radioactivity in dimers) + (0.7 × the radioactivity in trimers]/total radioactivity recovered). This value is within the range observed in other gram-negative peptidoglycans. In another strain of *L. pneumophila* grown in a different medium and analyzed by a different technique, Amano and Williams obtained results that were interpreted as suggesting a much higher degree of peptidoglycan cross-linking (1).

Philadelphia strain 1 had reasonably low MICs and MBCs for several β-lactam antibiotics, and the cells were sensitive to both the cidal and lytic effects of those agents. These findings together support the notion that the relative resistance of *L. pneumophila* infections to β-lactam antibiotics (2, 6, 9) may be primarily related to the peculiar mode of in vivo growth of this bacterium in phagosomes (12).

We did observe one unusual feature in the response of *L. pneumophila* to β-lactam antibiotics. Treatment of *L. pneumophila* cultures with penicillin caused a rapid loss of viability and lysis of bacteria (as determined by turbidity drop and release of labeled nucleic acids into the medium), but lysis was not accompanied by cell wall degradation. There was no significant decrease of the SDS-insoluble [^{3}H]glucosamine-labeled cell wall material during penicillin exposure, indicating that the peptidoglycan network remained as a large polymer (Fig. 2). In contrast, similar antibiotic treatments of pneumococci or *E. coli* resulted in the loss of 60 to 80% of the original SDS-insoluble radioactively labeled cell wall material (results not shown). The morphological appearance of *L. pneumophila* after treatment with penicillin also suggested the relative integrity of the murein sacculus despite a general "fading" of cytoplasm. On the other hand, the cell envelope of *E. coli* became fragmented and disrupted after exposure to penicillin (14). Possibly, the β-lactam antibiotic-induced lysis of *L. pneumophila* involves a murein hydrolase that nicks but does not degrade the peptidoglycan. In this respect, it is interesting that treatment of the *L. pneumophila* cell wall with egg white lysozyme has been reported to yield virtually quantitative cleavage of the glycan to disaccharides without solubilization of the wall (1).

It has been shown repeatedly that the physiological effects of β-lactam antibiotics on microorganisms vary greatly with the target organism. In tolerant strains of *Streptococcus sanguis* which lack autolytic activity, treatment with penicillin causes inhibition of growth but no lysis, no cell wall degradation, and only very slow loss of viability. In *Streptococcus pyogenes*, penicillin causes a halt in growth and rapid loss of viability but no lysis or cell wall degradation (11). In *E. coli* (14) or pneumococci (18), treatment with inhibitors of cell wall synthesis causes rapid killing, cell lysis, and cell wall degradation as well. The novel type of cell lysis without cell wall degradation, as described in this report for *L. pneumophila*, appears to be yet another example of the variation in the physiological responses of bacteria to β-lactam antibiotics.

ACKNOWLEDGMENTS

We acknowledge the assistance of Thomas Dougherty.

These investigations were supported by a Fellowship in Infectious Diseases from Cornell University Medical School (to S.W.).

LITERATURE CITED

1. **Amano, K., and J. C. Williams.** 1983. Peptidoglycan of *Legionella pneumophila*: apparent resistance to lysozyme hydrolysis correlates with a high degree of peptide cross-linking. J. Bacteriol. **153**:520–526.
2. **Bacheson, M. A., H. M. Friedman, and C. E. Benson.** 1981. Antimicrobial susceptibility of intracellular *Legionella pneumophila*. Antimicrob. Agents Chemother. **20**:691–692.

3. **Blundell, J. K., G. R. Smith, and H. R. Perkins.** 1980. The peptidoglycan of *Neisseria gonorrhoeae*: *O*-acetyl groups and lysozyme sensitivity. FEMS Microbiol. Lett. **9**:259–261.
4. **Buchanan, C. E., and J. L. Strominger.** 1976. Altered penicillin-binding components in penicillin-resistant mutants of *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. **73**:1816–1820.
5. **Dougherty, T. J.** 1983. Peptidoglycan biosynthesis in *Neisseria gonorrhoeae* strains sensitive and intrinsically resistant to β-lactam antibiotics. J. Bacteriol. **153**:429–435.
6. **Edelstein, P. H., and R. D. Meyer.** 1980. Susceptibility of *Legionella pneumophila* to twenty antimicrobial agents. Antimicrob. Agents Chemother. **18**:403–408.
7. **Feeley, J. C., G. W. Gorman, R. E. Weaver, D. C. Mackel, and H. W. Smith.** 1978. Primary isolation media for Legionnaires disease bacterium. J. Clin. Microbiol. **8**:320–325.
8. **Flesher, A. R., S. Ito, B. J. Monsheim, and D. L. Kasper.** 1979. The cell envelope of the Legionnaire's bacterium—morphologic and biochemical characteristics. Ann. Intern. Med. **90**:628–631.
9. **Fraser, D. W., C. Bapp, I. I. Wachsmuth, S. C. Feeley, and T. F. Tsai.** 1978. Antibiotic treatment of guinea pigs infected with agents of Legionnaire's disease. Lancet **i**:175–177.
10. **Gmeiner, J., and H.-P. Kroll.** 1981. Murein biosynthesis and *O*-acetylation of *N*-acetylmuramic acid during the cell-division cycle of *Proteus mirabilis*. Eur. J. Biochem. **117**:171–177.
11. **Horne, D., and A. Tomasz.** 1977. Tolerant response of *Streptococcus sanguis* to beta-lactams and other cell wall inhibitors. Antimicrob. Agents Chemother. **11**:888–896.
12. **Horwitz, M. A., and C. A. Silverstein.** 1980. Legionnaire's disease bacterium (*Legionella pneumophila*) multiplies intracellularly in human monocytes. J. Clin. Invest. **66**:441–450.
13. **Keel, J. A., W. R. Finnely, and J. C. Feeley.** 1979. Fine structure of the Legionnaire's bacterium—*in vitro* and *in vivo* studies of four isolates. Ann. Intern. Med. **90**:652–656.
14. **Kitano, K., and A. Tomasz.** 1979. Triggering of autolytic cell wall degradation in *Escherichia coli* by beta-lactam antibiotics. Antimicrob. Agents Chemother. **16**:838–848.
15. **Lacks, S., and R. D. Hotchkiss.** 1960. A study of the genetic material determining an enzyme activity in pneumococcus. Biochim. Biophys. Acta **106**:564–576.
16. **Spratt, B. G.** 1977. Properties of the penicillin binding proteins of *Escherichia coli*. Eur. J. Biochem. **72**:341–352.
17. **Tomasz, A., J. D. Jamieson, and E. Ottolenghi.** 1964. The fine structure of *Diplococcus pneumoniae*. J. Cell Biol. **22**:453–480.
18. **Tomasz, A., and S. Waks.** 1975. Mechanism of action of penicillin: triggering of the pneumococcal autolytic enzyme by inhibitors of cell wall synthesis. Proc. Natl. Acad. Sci. U.S.A. **72**:4162–4166.
19. **Warren, W. J., and R. J. Miller.** 1979. Growth of Legionnaires disease bacterium (*Legionella pneumophila*) in chemically defined medium. J. Clin. Microbiol. **10**:50–55.